## AMENDMENTS

### Amendments to the Specification

1. Please replace paragraph [page 2, lines 1-9] with the following paragraph:

   The tetanus and botulinum toxins are among the most lethal substances known to man, having a lethal dose in humans of between 0.1 ng and 1 ng per kilogram of body weight. Tonello et al., *Adv. Exp. Med. & Biol.* 389:251-260 (1996). Both toxins function by inhibiting neurotransmitter release in affected neurons. The tetanus neurotoxin (Te[NT]Tx)acts mainly in the central nervous system, while botulinum neurotoxin (BoNT) acts at the neuromuscular junction and other cholinergic synapses in the peripheral nervous system; both act by inhibiting neurotransmitter release from the axon of the affected neuron into the synapse, resulting in paralysis.

2. Please replace paragraph [page 2, lines 10-18] with the following paragraph:

   The tetanus neurotoxin (Te[NT]Tx) is known to exist in one immunologically distinct type; the botulinum neurotoxins (BoNT) are known to occur in seven different immunogenic types, termed BoNT/A through BoNT/G. While all of these types are produced by isolates of *C. botulinum*, two other species, *C. baratii* and *C. butyricum* also produce toxins similar to /F and /E, respectively. See *e.g.*, Coffield *et al.*, *The Site and Mechanism of Action of Botulinum Neurotoxin in Therapy with Botulinum Toxin* 3-13(Jankovic J. & Hallett M. eds. 1994), the disclosure of which is incorporated herein by reference.

3. Please replace paragraph [page 2, lines 19-26] with the following paragraph:

   Regardless of type, the molecular mechanism of intoxication appears to be similar. In the first step of the process, the toxin binds to the presynaptic membrane of the target neuron through a specific interaction between the heavy (H) chain and a cell surface receptor; the receptor is thought to be different for each type of botulinum toxin and for Te[NT]Tx. Dolly *et al.*, *Seminars in Neuroscience* 6:149-158 (1994), incororated by reference herein. The carboxyl terminus of the heavy chain appears to be important for targeting of the toxin to the cell surface. *Id.*

4. Please replace paragraph [page 3, lines 11-22] with the following paragraph:

   The last step of the mechanism of botulinum toxin activity appears to involve reduction of the disulfide bond joining the H and light (L) chain. The entire toxic activity of botulinum and tetanus toxins is contained in the L chain of the holotoxin; the L chain is a zinc ($Zn^{++}$) endopeptidase which selectively cleaves proteins essential for recognition and docking of neurotransmitter-containing vesicles with the cytoplasmic surface of the plasma membrane, and fusion of the vesicles with the plasma membrane. T[xNT]eTx, BoNT/B BoNT/D, BoNT/F, and BoNT/G cause degradation of synaptobrevin (also called vesicle-associated membrane protein (VAMP)), a synaptosomal membrane protein. Most of the cytosolic domain of VAMP extending from the surface of the synaptic vesicle is removed as a result of any one of these cleavage events. Each toxin (except Te[N]Tx and BoNT/B) specifically cleaves a different bond.

5. Please replace paragraph [page 4, lines 5-13] with the following paragraph:

Both Te[N]Tx and BoNT are taken up at the neuromuscular junction. BoNT remains within peripheral neurons, and blocks release of the neurotransmitter acetylcholine from these cells. Through its receptor, Te[N]Tx enters vesicles that move in a retrograde manner along the axon to the soma, and is discharged into the intersynaptic space between motor neurons and the inhibitory neurons of the spinal cord. At this point, Te[N]Tx binds receptors of the inhibitory neurons, is again internalized, and the light chain enters the cytosol to block the release of the inhibitory neurotransmitters 4-aminobutyric acid(GABA) and glycine from these cells.

6. Please replace paragraph [page 17, lines 10-16] with the following paragraph:

In another aspect, the invention comprises a modified clostridial neurotoxin derived from tetanus toxin (Te[NT]Tx), or one or more of the botulinum toxin (BoNT) subtypes in which the naturally-occurring interchain loop region has been replace with a modified loop region comprising a different amino acid sequence conferring 1) resistance to cleavage by host proteases or autolytic action, and/or 2) lability to a selected protease. Preferably the cleavage site is highly specific for the selected protease.

7. Please replace paragraph [page 20, lines 17-25] with the following paragraph:

In a preferred embodiment, the therapeutic element is a polypeptide comprising a clostridial neurotoxin light chain or a portion thereof retaining the SNARE-protein sequence-specific endopeptidase activity of a clostridial neurotoxin light chain. The amino acid sequences of the light chain of botulinum neurotoxin (BoNT) subtypes A-G have been determined, as has the amino acid sequence of the light chain of the tetanus neurotoxin (Te[NT]Tx). Each chain contains the $Zn^{++}$-binding motif His-Glu-X-X-His SEQ ID NO: 17 (N terminal direction at the left) characteristic of Zn++-dependent endopeptidases (HELIH SEQ ID NO: 25 in Te[NT]Tx, BoNT/A /B and /E; HELNH SEQ ID NO: 26 in BoNT/C; and HELTH SEQ ID NO: 27 in BoNT/D).

8. Please replace paragraph [page 20, lines 26-27 through page 21, lines 1-15] with the following paragraph:

Recent studies of the BoNT/A light chain have revealed certain features important for the activity and specificity of the toxin towards its target substrate, SNAP-25. Thus, studies by Zhou *et al. Biochemistry* 34:15175-15181 (1995) have indicated that when the light chain amino acid residue $His_{227}$ is substituted with tyrosine, the resulting polypeptide is unable to cleave SNAP-25; Kurazono *et al., J. Biol. Chem.* 14721-14729 (1992) performed studies in the presynaptic cholinergic neurons of the buccal ganglia of *Aplysia californica* using recombinant BoNT/A light chain that indicated that the removal of 8 N-terminal or 32 C-terminal residues did not abolish toxicity, but that removal of 10 N-terminal or 57 C-terminal residues abolished toxicity in this system. Most recently, the crystal structure of the entire BoNT/A holotoxin has been solved; the active site is indicated as involving the participation of $His_{222}$, $Glu_{223}$, $His_{226}$, $Glu_{261}$ and $Tyr_{365}$. Lacy *et al., supra.* (These residues correspond to $His_{223}$, $Glu_{224}$, $His_{227}$, $Glu_{262}$ and $Tyr_{366}$ of the BoNT/A L chain of Kurazono *et al., supra.*) Interestingly, an alignment of BoNT/A through E and Te[N]Tx light chains reveals that every such chain invariably has these residues in positions analogous to BoNT/A. Kurazono *et al., supra.*

9. Please replace paragraph [page 14, lines 1-4] with the following paragraph:

Figure 3B shows the results of an experiment in which affinity purified recombinant single-chain (SC) TeTx is nicked with enterokinase, then separated using SDS-PAGE under reducing and non-reducing conditions and subjected to a Western blot using anti Te[T%X]Tx heavy chain antibody.

10. Please replace paragraph [page 23, lines 24-27 through page 24, lines 5] with the following paragraph:

Alternatively, and most preferably, the binding tag may comprise some or all of the amino acid sequence of an appropriately chosen polypeptide coexpressed with the single chain toxin as a fusion protein; such polypeptides may comprise, without limitation, the maltose binding domain of maltose binding protein (MBP); a $His_6$ tag (a run of 6 histidine residues); the calmod[i]ulin binding domain of calmodulin binding protein; and the glutathione binding domain of glutathione-S-transferase. Other polypeptide binding tags are well known to those of skill in the art, and can easily be adapted for use in the present invention.

11. Please replace paragraph [page 18, lines 13-23] with the following paragraph:

Any non-human protease recognizing a relatively rare amino acid sequence may be used, provided that the amino acid recognition sequence is also known. Examples of proteases to be selected as activators may include any of the following, without limitation: bovine enterokinase, plant proteases such as papain (from *Carica papaya*)and legumain, insect papain homolog (from the silkworm *Sitophilus zeamatus*), and crustacian papain homolog (decapod), Tobacco etch virus (TEV) protease, which cleaves the ~~sequence EXXYXQS/G~~ sequences EXXYXQS (SEQ ID NO: 22) and EXXYQG (SEQ ID NO: 23); GENENASE® from *Bacillus amyliquifaciens*, which cleaves the sequence HY or YH; and PRESCISSION® protease from human rhinovirus 3C, which cleaves the amino acid sequence LEVLFQGP SEQ ID NO: 16. As used above, the letter X indicates any amino acid.

12. Please replace paragraph [page 27, lines 18-25] with the following paragraph:

The nucleotide sequence encoding the wild-type TeTx L chain is obtained from plasmid pMAL-LC, described in Li *et al.*, *Biochemistry* 33, 7014-7020 (1994), incorporated by reference herein. The plasmid encodes the TeTx light chain as a fusion protein with maltose binding protein (MBP) located immediately upstream of the coding sequence for the L chain. The MBP and L chain portions of the fusion protein are designed to contain the cleavage site for human blood coagulation factor Xa (Ile-Glu-Gly-Arg SEQ ID NO: 28) to facilitate removal of the MBP once affinity purification has been performed.

13. Please replace paragraph [page 37, lines 17-25] with the following paragraph:

In the recombinant single-chain TeTx, the LC and HC are linked by 17 amino acids (GEKLYDDDDKDRWGSSR SEQ ID NO: 29), followed by the beginning of the H chain sequence (~~SLTDLGGEL . . .~~ SEQ ID NO: 20). N-terminal amino acid sequencing of the larger fragment produced by both trypsin and Arg C protease reveal that first 5 amino

acids of the 100 kDa trypsin and Arg C protease cleavage product protein are SLTDL of SEQ ID NO: 20; thus, these proteases appear to cleave the single—chain toxin between the R-S bond (see Figure 1) so as to liberate the H chain and the L chain containing the EK linker at its C terminus, with this variant therefore yielding a dichain toxin essentially identical to the EK nicked toxin.

14. Please replace paragraph [page 5, lines 9-21] with the following paragraph:

Despite the clear therapeutic efficacy of clostridial neurotoxin preparations, industrial production of the toxin is difficult. Production of neurotox[o]in from anaerobic Clostridium cultures is a cumbersome and time-consuming process including a multi-step purification protocol involving several protein precipitation steps and either prolonged and repeated crystallisation of the toxin or several stages of column chromatography. Significantly, the high toxicity of the product dictates that the procedure must be performed under strict containment (BL-3). During the fermentation process, the folded single-chain neurotoxins are activated by endogenous clostridial proteases through a process termed nicking. This involves the removal of approximately 10 amino acid residues from the single-chain to create the dichain form in which the two chains remain covalently linked through the interchain disulfide bond.

15. Please replace paragraph [page 17, lines 17-27 through page 18, lines 1-2] with the following paragraph:

The interchain loop region of certain clostridial neurotoxins, for example, BoNT/E, is naturally resistant to proteolytic cleavage *in vivo*. This protease resistance may reflect a secondary or tertiary structure that makes the loop more resistant to indigenous proteases than other clostridial neurotoxins. In one embodiment of the present invention, therefore, the inter-chain loop region of BoNT/E is substituted for the natural loop region occurring [an] in another BoNT having greater therapeutic activity or duration of action, for example BoNT/A or /B. In another embodiment of the invention the loop region of BoNT/E is modified to contain a proteolytic cleavage site highly specific to a selected protease prior to the subcloning. The otherwise highly conserved BoNT/E loop region would be resistant to indigenous proteases, or those encountered within a human, but would retain the ability to be activated by digestion with the selected protease.

16. Please replace paragraph [page 28, lines 11-26] with the following paragraph:

The DNA fragment containing the H chain is obtained from plasmid pMAL-HC; construction of this vector is described in Li et al., *J. Biochem.* 125:1200-1208(1999), hereby incorporated by reference herein. Briefly, the gene encoding the H chain is constructed by assembling three DNA fragments containing different portions of the H chain coding sequence which had been cloned into separate plasmids. The fragments comprising the amino terminal half of the H are first amplified using standard polymerase chain reaction methods (see, e.g., Mullis, U.S. Patent No. 4,683,202 and Mullis et al., U.S. Patent No. 4,800,159, both incorporated by reference herein in their entirety) and the following primers: PCR primers a (containing a Xba I cleavage site) and b (containing a Bgl II cleavage site) (SEQ ID NO: 3 and 4, respectively) are used to amplify the H chain fragment contained in a plasmid termed pTet8; PCR primers c (containing a Bgl II cleavage site) and d (containing both a Hind III and a Sal I cleavage

site)(SEQ ID NO: 5 and 6, respectively) are used to amplify the H chain fragment contained in a plasmid termed pTet14. The nucleotide sequences of these primers are provided below, with restriction sites underlined.

17. Please replace paragraph [page 47, lines 21-27 through page 28, lines 1-5] with the following paragraph:

The PCR product was digested with BamHI and HindIII, and the digest subjected to agarose gel electrophoresis. Staining of the agarose gel with ethidium bromide revealed a major DNA fragment of approximately 3.5 kilobases (see Fig. 10). The band containing this fra[n]gment was excised from the gel, and the DNA purified from the agarose and ligated to BamHI and HindIII-cut pQE30 vector (Qiagen). The resulting ligated plasmid was used to transform *E. coli* strain JM 109 as described above, and the transformants plated onto selective LB agar plates. Several clones were recovered and the presence of the correct BoNT/E DNA insert checked by restriction digest. The resultant construct contains the BoNT/E gene (minus the first methionine) fused to the $His_6$ tag of the pQE30 vector, and contains 2 extra amino acid residues (glycine, serine), which are contributed by the engineered BamHI site.

18. Please replace paragraph [page 49, lines 23-26 through page 50, lines 1-2] with the following paragraph:

The *E. coli* strain M15 (Qiagen) was used for expression of the BoNT/E single-chain construct. This strain carries an endogenous plasmid (pREP4, kanamycin resistant) containing a region encoding the lac $I^q$ repressor gene in order to prevent transcription of the neurotoxin gene prior to induction with IPTG. The pQE30 vector contains a T5 bacteriophage RNA polymerase promoter, which is also recognized by *E. coli* RNA polymerase.

19. Please replace paragraph [page 34, lines 1-11] with the following paragraph:

Figure 3 shows the results of a second experiment, in which affinity purified recombinant single-chain (SC) TeTx is nicked with enterokinase as follows. Thirty micrograms of purified single chain toxin are incubated with 1 unit of enterokinase in a solution containing 50 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 0.1% ~~Tween~~TWEEN-20®(v/v) (polyoxyethylene (20) sorbitan monolaureate). As a control, the recombinant protein is incubated in the same reaction mixture containing no EK. These samples, plus an aliquot of native (non-recombinant) TeTx are subjected to SDS-PAGE in an 8% polyacrylamide gel under either reducing (+BME) or non-reducing (-BME) conditions. The resulting gel is used both for a Western blot and subsequent detection using anti-H claim antibodies (Fig 3B), and direct staining with Coomassie Blue (Fig 3A).

20. Please replace paragraph [page 50, lines 9-22] with the following paragraph:

Subsequent centrifugation of the culture yielded ~ 2.3 g of wet cell pellet which was resuspended in 10 ml of extraction buffer (20 mM Hepes pH 7.0, 300 mM NaCl, 5mM benzamidine, 2 μM pepstatin and 2 μM E-64). Lysozyme was added to a final concentration of 0.25 mg/ml, and the cell suspension incubated on ice for 60 minutes. Approximately 0.5 ml of glass beads (0.1 mm diameter from Biospec) was added to the cell suspension, followed by vortexing for 2 minutes to break the cells. Cell-free extracts

was obtained by centrifugation at 10,000xg for 30 minutes at 4 °C. The supernatant was incubated with 0.5 ml of ~~Talon~~TALON® cobalt metal affinity agarose resin (Clontech) pre-washed with extraction buffer in a rocking platform for 45 minutes at 4°C. The resin was then loaded into a disposable chromatography column and washed twice with 10 bed volumes of wash buffer (20 mM Hepes pH 7.0, 300 mM NaCl, 2 mM imidazole) before eluting the bound neurotoxin in 6 bed volumes of elution buffer (20 mM Hepes pH 7.0, 300 mM NaCl, 150 mM imidazole).